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(57) Abstract

Modified plants are disclosed which have enhanced starch-producing capability as a result of a change in the concentration of one or more of the metabolites which take part in glycolysis and the TCA cycle in the plant. The effect may be achieved for example by a genetic modification to reduce the activity of NAD-malic enzyme.

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TRANSGENIC PLANTS HAVING INCREASED STARCH CONTENT

This invention relates to modified plants having enhanced starch-producing capability and to plant cells capable of regeneration into such plants. The invention also relates to methods for producing increased quantities of starch and to recombinant DNA molecules for transforming plants.

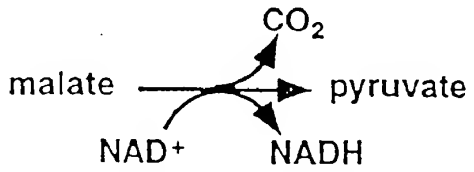
Starch is the major storage carbohydrate found in higher plants, and as such represents the largest single energy-source in the human diet globally. In addition to being a major foodstuff in its own right, the physical properties of starch (gel and colloid formation) result in usage as a natural additive to processed foods primarily as a thickening agent. There are also important non-food uses of starch, as a component of adhesive, in the paper and textiles industries. In the latter case the physical properties of starch are particularly crucial. Other industries using starch are the plastics and pharmaceutical industries; in the latter it is used e.g. as an inert carrier. The physical properties of starch are in part dependent on the ratio of amylose (long straight glucan chains) to more highly branched amylopectin. In recent years there has been much interest in the manipulation of starch quantity and quality by the biotechnology industry. In particular a major aim has been the production of genetically modified plant varieties that contain increased starch content, or starch which is structurally distinct from that found naturally. As a result of the easy application of transgenic technologies to potato, most of the work to date has focused on this species.

Manipulation of starch quality has met with some success. For example, while manipulation of endogenous starch biosynthetic enzyme activities has only small effects on yield, there are often dramatic changes in the physical properties of the starch produced (Müller-Röber *et al* 1994); these changes are not well understood from a mechanistic

perspective. Recently expression of a bacterial starch branching enzyme in an amylose-free variety of potato has resulted in the production of amylopectin with a very high degree of branching (Kortstee *et al* 1996). Many of the variations in starch structure currently being produced have potential commercial uses.

Approaches to increasing starch yield have met with only limited success. Over-expression of a range of genes encoding starch biosynthetic enzymes, either from potato itself or other species, has not resulted in increased tuber starch contents (Müller-Röber *et al* 1994). Limited success has been achieved by expressing a mutated bacterial gene encoding an unregulated form of ADP-glucose pyrophosphorylase, which resulted in a modest increase in tuber starch content of around 20% (Stark *et al* 1992), but recent work repeating this approach in a higher yielding potato variety has failed to demonstrate an increase in starch content, under either field or glasshouse conditions (Herbers *et al* 1996). In conclusion, there is still much scope for increasing the tuber starch content of transgenic potato plants.

NAD-malic enzyme (NAD-ME) is a metabolic enzyme ubiquitous in higher plants, but its function is currently a matter of debate. It catalyses the oxidative decarboxylation of malate:



In full, this enzyme is NAD-linked malic dehydrogenase (decarboxylating) (non-OAA decarboxylating) enzyme, enzyme classification EC 1.1.1.39. It will be referred to herein as NAD-ME.

As part of a general investigation into the control of flux through the tricarboxylic acid (TCA) cycle (see Figure 1), cDNA clones encoding NAD-ME subunits were isolated; in potato, there are two NAD-

ME subunits of 62 kDa and 59 kDa respectively (Winning *et al* 1994). In order to better understand the function of NAD-ME, transgenic potato plants expressing the cDNA clones encoding the 59 kDa subunit in the antisense orientation were produced, with the overall aim of reducing the NAD-ME activity *in planta* and determining the metabolic consequences (Hill *et al* 1996). Preliminary studies indicated that these transgenic plants have reductions in NAD-ME activity down to 40% of that found in the wild-type, but show no obvious morphological phenotype or alteration in growth rate.

It has now been discovered that by reducing NAD-ME activity in potato plants, the tuber starch content of the plants is significantly increased. NAD-ME is localised in the mitochondria and hence is metabolically distinct from the pathway of starch synthesis (see Figure 1) which goes on in the amyloplasts of potato tubers. The effect observed on starch synthesis is mediated through a change in one or more metabolite concentrations. This provides a new approach for increasing starch content. As already noted, previous attempts to manipulate starch synthesis have targeted the starch biosynthetic enzymes themselves. Once a metabolite whose concentration is altered by the reduction in NAD-ME activity and whose altered concentration leads to an increase in starch content, has been identified, it is possible to devise alternative strategies to manipulate the concentration of the metabolite and thus starch content. It has in fact been shown that an increased 3-phosphoglyceric acid (3-PGA) content correlates with an increase in starch content. This is expected to be the case for other metabolites. The identification of further metabolites and manipulation of their concentrations are within the capability of a person skilled in the art.

The present invention therefore provides in one aspect a method for producing starch comprising the steps of:

a) providing a plant, which plant is modified so as to have increased starch content as a result of a change in the concentration of one or more of the metabolites which are intermediates in glycolysis and the TCA cycle in the plant; and

b) harvesting starch-containing material from the plant.

In another aspect the invention provides both a modified plant having enhanced starch-producing capability as a result of a change in the concentration of one or more of the metabolites which are intermediates in glycolysis and the TCA cycle in the plant, with the proviso that potato plants having reduced NAD-ME activity by means of antisense techniques are excluded; and a modified plant cell, genetically modified so as to be capable of regeneration into the modified plant.

In further aspects, the invention provides the use of a modified plant as described, for the production of starch-containing material; and the use of a modified plant cell as described, for regenerating a plant for the production of starch-containing material.

In further aspects, the invention provides the use of a starch-storing plant which is genetically modified so as to decrease NAD-ME activity, for the production of starch-containing material; and the use of a plant cell of a starch-storing plant, said cell genetically modified so as to decrease NAD-ME activity in a plant regenerated from the plant cell, for regenerating a plant for the production of starch-containing material.

In another aspect, the invention provides a recombinant double-stranded DNA molecule comprising a promoter which functions in plants, which promoter is capable of functioning preferentially in starch-storing tissues of plants, said promoter operatively linked to a DNA sequence capable of reducing NAD-ME activity in plants.

In further aspects the invention provides a plant cell transformed with the recombinant DNA molecule described; and a plant

consisting of such plant cells, wherein NAD-ME activity is preferentially decreased in starch-storing tissues of the plant.

In the attached figures:

Figure 1A shows interactions between glycolysis and the TCA cycle, and starch biosynthesis in starch-storing plants;

Figure 1B shows the steps of glycolysis in full;

Figure 2 shows NAD-ME activity in transgenic potato lines carrying the cDNA encoding the 59 kDa subunit of NAD-ME in the antisense orientation, (A) under the control of the cauliflower mosaic virus 35S promoter and (B) the B33 patatin promoter;

Figure 3 shows starch content and starch synthetic flux in tubers of transgenic potato plants expressing the cDNA encoding the NAD-ME in the antisense orientation under the control of the cauliflower mosaic virus 35S promoter;

Figure 4 shows starch content in tubers of transgenic potato plants expressing a cDNA encoding NAD-ME in the antisense orientation under the control of the cauliflower mosaic virus 35S promoter.

Figure 5 shows the relationship between NAD-ME activity and starch content in developing potato tubers;

Figure 6 shows the metabolism of ^{14}C-glucose by tuber discs from developing tubers with reduced NAD-ME activity; and

Figure 7 shows the relationships between NAD-ME activity and 3-PGA content, and 3-PGA content and starch content, in developing potato tubers in wild type and transgenic plants with reduced NAD-ME activity.

Figure 8 shows cDNA sequences encoding potato NAD-ME 59kDa and 62 kDa subunits (Winning *et al* 1994; Database Accession Nos: Z23002 and Z23023, respectively).

Interactions between the TCA cycle and starch biosynthesis in developing potato tubers are shown in Figure 1A. NAD-ME catalyses

the conversion of malate to pyruvate in the TCA cycle, the enzymes for which are located inside the mitochondria. Malate and pyruvate, both of which enter the TCA cycle, are products of the process of glycolysis (Figure 1B), which involves the degradation of hexose sugars. Metabolites earlier in the chain than malate and pyruvate in glycolysis include oxaloacetate (OAA), phospho(enol)pyruvate (PEP) and 3-phosphoglycerate (3-PGA). Of these PEP and 3-PGA are known to have a positive effect on starch synthesis by activating ADP-glucose pyrophosphorylase (AGPase) (Preiss 1988). When NAD-ME activity is deceased, a build up of one or more of these metabolites, including 3-PGA, leads to an increase in starch synthesis.

The metabolites which are intermediates in glycolysis and the TCA cycle are considered to be all of the metabolites from fructose-6-phosphate in the schemes in Figure 1A and 1B, through to and including metabolites in the TCA cycle.

The metabolites and enzymes which take part in glycolysis in potato tubers are shown in Figure 1A and in detail in Figure 1B. The same metabolites are involved in glycolysis in all organisms (see for example Dennis *et al.* 1996). The enzymes involved are also the same in that they catalyse the same reactions, but they are different in the sense that their amino acid sequences are different (although related) in each organism. Enzymes from different organisms catalysing the same reaction can thus be described as analogous.

Similarly, the metabolites and enzymes which take part in the TCA cycle are the same in all higher plants (see for example Hill 1996).

The plants with which the invention is concerned may be any higher plants which store starch, either permanently or transiently. Generally, the plants will have starch-storing tissues which may be organs such as tubers, roots, seeds and grains. Other starch-storing tissues include for example leaves and stems in forage crops such as maize,

grasses and alfalfa. Starch-storing tissues include tissues which store starch transiently, for example oil-storing and protein-storing tissues such as seeds. Modified plants according to the invention may thus be useful for the production of starch, in which case starch-containing material is harvested, or they may be useful for the production of other materials whose production is increased in the presence of modified starch synthesis. Such other materials include for example oils and fibres from crop plants such as oil seed rape and flax, respectively.

Plants with which the invention is concerned include the following groups of plants:

(i) fruits and vegetables (e.g. potato, tomato, banana);

(ii) cereals (e.g. maize (large grain cereal), and wheat, rice and barley (small grain cereals));

(iii) oilseeds (e.g. soya, oilseed rape, sunflower).

Other higher plants which do not fall into any of the above three groups are also included, for example fibre crops such as trees and flax.

The plants with which the invention is concerned may be either monocots or dicots. Specific tissues in which it may be desirable to increase starch synthesis include maize endosperm, rice endosperm, pea endosperm, wheat endosperm, soy bean cotyledons, potato tubers and cassava tubers. Examples of commercially useful plants to which the invention may be applied include, in addition to potato and other tuberous crops: maize, wheat, rice, barley, sorghum, millet, oil seed rape, tomato, sugar beet, cassava, yam, turnip, swede, carrot, banana, and legumes such as peas and beans. The commercial use of each of these plants depends at least in part upon the starch content or the starch-producing capability of the plant.

Starch synthesis in plants is generally localised in organelles called plastids, which include the chloroplasts in photosynthetic tissues,

and the amyloplasts in non-photosynthetic tissues. Starch synthesis also occurs in the leucoplasts of oil-storing tissues.

It may be beneficial to target the increased starch content preferentially to starch-storing tissues in the plant. This would enable any deleterious effects on photosynthesis to be avoided. The expression "starch-storing tissues" is intended to cover tissues that store starch permanently, or transiently on a long or short-term basis. Starch-storing tissues are generally non-photosynthetic tissues, thus e.g. leaves are excluded. Certain starch-storing tissues may however have a photosynthetic phase in their development. Bananas, for example, are green (photosynthetic) when unripe, and store starch until ripening, when the starch is converted to sugar. Tomatoes also photosynthesize when unripe; starch is stored on a short-term transient basis, acting as a buffer for sugar content.

The increase in starch content is achieved in the plant by genetic manipulation and as such is preferably an inheritable feature. Preferably, the plant has genetic material stably incorporated into its genome which genetic material is capable of leading to enhanced starch production. In a particular embodiment of the invention described herein, potato plant cells are transformed with a double-stranded cDNA molecule corresponding to a NAD-ME subunit gene in the antisense orientation, under the control of a promoter. Results indicate that a 50% reduction in NAD-ME activity is required to achieve significantly increased starch content in potatoes. Therefore, it may be preferable to reduce NAD-ME activity by at least 50%. That threshold may vary according to the particular species of plant and the particular tissue concerned, and can be determined in a straight-forward manner by experimentation.

A double-stranded DNA molecule for inserting into a plant cell genome to produce a plant having increased starch content, may be introduced into the plant cell by any suitable method. Suitable vectors for

transferring genes into plants are well known. The two most widely-used methods for transforming plant cells are *Agrobacterium*-mediated transfer (used herein in the Examples), and direct delivery of DNA by microprojectile bombardment (the "gene gun") or using silicon carbide whiskers. Techniques for regenerating plants from transformed plant cells are also known. Where the introduced or heterologous DNA becomes a *bona fide* genetic locus that behaves in a Mendelian fashion, this is often referred to as a "transgene".

For expressing a heterologous DNA molecule in a plant cell, a suitable promoter is required. A suitable promoter is one which functions in the plants concerned; suitable promoters are known to those skilled in the art. The promoter may be an essentially constitutively active promoter such as the cauliflower mosaic virus 35S promoter rice actin promoter, Arabidopsis ubiquitin (UBQ3) promoter, or maize ubiquitin promoter, or it may be a conditional promoter which is preferentially active under certain conditions. Conditional promoters include promoters which are preferentially active in certain tissues, for example the patatin promoter which gives tuber-enhanced gene expression in the potato. The patatin promoter also functions in tomato, where it is effectively fruit-specific. The patatin promoter is sugar induced. Other tuber-enhanced promoters include the potato GBSS/waxy promoter. Examples of grain/seed-enhanced promoters include the maize zen promoter and promoters from starch biosynthetic genes. Fruit-enhanced promoters include the tomato polygalac turonase (PG) promoter.

When introducing a DNA molecule into a cell there are various factors which will need to be taken into consideration in order to achieve the desired effect, usually expression of the DNA in the transformed cell. The location of the transgene in the cell genome will affect its expression, as will the number of copies of the transgene present in the cell genome. Different promoters can have different strengths, and

thus the rate of transcription of the transgene DNA into RNA can vary. Promoter strength therefore affects expression of the transgene.

In addition, factors such as the stability of a transgene product need consideration, in particular where the heterologous DNA is not native to the species into which it is to be introduced. Where the DNA is introduced for the purpose of protein expression, the DNA may be modified in that known mRNA instability motifs and/or fortuitous splice regions are removed, or codons which are preferred by the organism into which the recombinant DNA is to be inserted are used, so that expression of a thus modified DNA in the said organism yields substantially similar proteins having a substantially similar activity or function to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein encoding components of the unmodified recombinant DNA are endogenous. Where antisense or cosuppression techniques are used, appropriate steps may be taken to enhance the stability of the RNA products responsible for inhibiting expression of the proteins for which reduced activity is desired. A person skilled in the art will be able to devise a suitable strategy for genetic manipulation taking into account all of these factors.

Four hypotheses were proposed concerning the effect of reduced NAD-ME activity on the accumulation of starch in potato tubers, three of which can be ruled out.

1. **Redirection of carbon**. Carbon enters developing tubers as sucrose, which is broken down into hexose phosphates. These hexose phosphates are then partitioned between three fates: synthesis of starch; synthesis of cellular constituents, primarily cellulose and other cell wall components for cell expansion; and respiration to provide the ATP required for biosynthesis. The first hypothesis is that a reduction in NAD-ME activity inhibits the flux of carbon into respiration, and the 'extra' carbon is diverted into starch synthesis. This hypothesis can be rejected for two reasons.

First, since the ATP required for starch synthesis is derived from respiration, a significant reduction in the rate of respiration should lead to a reduction, rather than an increase, in the rate of starch synthesis. Second, no effect of the transgenic manipulation on the rate of respiration has been detected.

2. **Induction of starch biosynthetic enzymes**. This hypothesis proposes that some change in a metabolite content, probably in a metabolite near to NAD-ME (e.g. an intermediate in the TCA cycle) is acting as a signal changing the expression of the genes encoding one or more of the starch biosynthetic enzymes. The latter change would be likely to lead to an increase in the rate of starch biosynthesis. This hypothesis has been tested by measuring the activity of four of the six unique steps of starch synthesis. No significant increase was found in the activity of phosphoglucomutase, ADP-glucose pyrophosphorylase (AGPase), inorganic pyrophosphatase or soluble starch synthase (see Figure 1).

3. **Increase in concentration of metabolites regulating starch synthesis**. Starch synthesis in plants is regulated by a number of metabolites. The major target for this regulation appears to be ADP-glucose pyrophosphorylase which is regulated by a number of metabolites at the lower end of glycolysis, particularly 3-phosphoglyceric acid (3PGA) and phospho(enol)pyruvic acid (PEP) (Preiss 1988). These molecules are activators of AGPase, and act antagonistically to inorganic phosphate, which is a negative regulator of this enzyme. It is possible that reduction of NAD-malic enzyme activity has led to a build up of malate, oxaloacetate (OAA), PEP and 3PGA (see Figure 1), with the increases in the latter two metabolites leading to an increase in the flux to starch synthesis. This hypothesis can be easily tested, by measuring the steady-state contents of metabolites in the transgenic lines. In fact, it has been tested and resulted in the demonstration that in transgenic potato lines with reduced NAD-ME activity, 3-PGA content correlates with starch content.

4. **Build up of glucose-1-phosphate**. Glucose-1-phosphate is the substrate of starch synthesis, so that if the reduction in NAD-malic enzyme activity causes a build up of all the intermediates of glycolysis, then the increased glucose-1-phosphate concentration may lead to an increased rate of starch synthesis simply through a substrate supply effect. This hypothesis is unlikely to be correct, since metabolite contents within cells are generally tightly buffered. Furthermore, no significant changes in glucose-1-phosphate levels were detectable in the transgenic lines.

Described below are various alternative strategies for achieving an increase in storage organ starch accumulation according to the invention. It will be understood that the following are given as examples to further illustrate the invention and are not intended to be limiting in any way.

a. Alternative approaches for the reduction in NAD-malic enzyme activity in potato.

i. Antisense reduction of the 62 kDa subunit, either alone or in combination with reduction in the 59 kDa subunit.

ii. Reduction of activity using co-suppression constructs. When coding sequences or fragments of coding sequences are reintroduced into the plant species from which they were isolated there is often a reduction in the activity of the enzyme encoded, through the phenomenon of co-suppression (see e.g. Gottlob-McHugh *et al* 1992 and Davies *et al* 1997). Thus, NAD-malic enzyme activity may be reduced by transforming potato with sequences from the genes encoding either or both of the subunits of NAD-malic enzyme in the sense orientation.

iii. Targeted gene disruption by homologous recombination (e.g. Kempin *et al* 1997) and excision/repair mechanisms ("Kimeroplasty", e.g. May *et al* 1997 and Kimiec *et al* 1997).

iv. Manipulation of the expression of endogenous NAD-malic enzyme genes. Genomic clones for both the subunits of NAD-malic enzyme have been isolated, and the promoter structure is being analysed. For any specific DNA binding proteins involved in the regulation of NAD-malic enzyme expression, it will be possible, through the manipulation of the levels of these protein through antisense or co-suppression, to reduce the expression of both subunits of NAD-malic enzyme.

v. NAD-malic enzyme functions as a heterodimer of the two subunits. As well as existing as the dimer it is believed that the tetramer and octamer are also found *in vitro* (Grover and Wedding 1984). The dimer, tetramer and octamer have distinct kinetic properties and it has been suggested that changes in aggregation state may be involved in regulating the activity of this enzyme *in vivo* (Grover and Wedding 1984), although there is no definitive evidence supporting this idea. It may be possible, using a 'two-hybrid' screening procedure in yeast (Fields and Song 1989) to isolate peptides that disrupt either the 59-62 interaction, or the formation of tetramers or octamers. The introduction of DNA sequences encoding these peptides into trangenic plants may provide a further method for reducing the activity of NAD-malic enzyme.

b. Approaches for the reduction of NAD-malic enzyme activity in starch-storing species.

An antisense approach, and the alternatives described in (a) can equally well be applied to any higher plant species, provided that cDNA and genomic clones encoding the subunits of NAD-malic enzyme can be isolated, and that a transformation system is available. The isolation of cDNA and genomic clones encoding NAD-malic enzyme subunits from other species is routine; PCR amplification, heterologous screening, and screening of expression libraries with antibodies which are

available or can be produced by known methods, are all expected to succeed. Transformation systems exist for many of the species that are of economic significance, including maize, and rice. It will be possible to develop transformation systems for other species for which they are not currently available. A major requirement is that a suitable promoter exists for driving expression of the transgenes in the required tissues; suitable promoters are generally available and are described in the literature.

c. Alternative approaches to increasing the starch content of storage organs.

As discussed above, NAD-malic enzyme is metabolically distant from the pathway of starch synthesis, and the effect on starch synthesis is mediated through a change in one or more metabolite concentrations. Alternative strategies to manipulate the concentrations of the affected metabolites can be devised. For example since starch synthesis is activated by an increase in 3-PGA as indicated above, a similar effect may be achieved by reducing the activity of cytosolic malate dehydrogenase, PEP carboxylase (PEPCase), or pyruvate kinase, or any of these in combination (see Figure 1). Alternatively, increasing the activity of phosphoglycerate kinase can be expected to lead to an increase in the concentration of 3-PGA. More dramatic changes could potentially be achieved by combining raised activity of phosphoglycerate kinase with reduced activity of NAD-malic enzyme, cytosolic malate dehydrogenase, PEPCase or pyruvate kinase. Manipulating the activities of phosphoglyceromutase or enolase may also be effective.

Since the increased starch synthesis observed in potato tubers with reduced NAD-malic enzyme activity is at least in part due to increased 3-PGA concentration, the plants and methods according to the invention may employ an increase in concentration of this metabolite. 3-PGA is an intermediate in the glycolytic sequence that occurs in the cytosol of all plant cells (Dennis, et al., 1996). A simple metabolic scheme of the

pathway is shown in Figure 1B The enzyme that produces 3-PGA is phosphoglycerate kinase and the enzyme that uses it as a substrate is phosphoglycerate mutase. In principle the concentration of 3-PGA could be increased by increasing the activity of any of the enzymes that precede 3-PGA in the glycolytic sequence (phosphofructokinase, pyrophosphate:fructose-6-phosphate 1-phosphotransferase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase) or by reducing the activity of any of the enzymes that follow 3-PGA (phosphoglycerate mutase, enolase, pyruvate kinase, PEP carboxylase, cyotsolic malate dehydrogenase), as well as enzymes found in the mitochondria such as NAD-malic enzyme. However, manipulation of the following enzymes to achieve enhanced starch synthesis can be ruled out:

1. Phosphofructokinase: Burrell, et al. (1994) produced transgenic potato plants that have up to a 20-fold increase in phosphofructokinase activity. In the tubers of these plants there is a considerable increase in the concentration of 3-PGA, but starch synthesis is unaltered. The most likely explanation for this is the reduction in the concentration of hexose phosphates in the tubers; in particular the glucose-1-phosphate concentration is reduced which will lead to a reduction in the rate of starch synthesis because it is the substrate for this pathway. The rate of starch synthesis in these plants is unaltered because the effects of reduced glucose-1-phosphate and increased 3-PGA cancel each other out.
2. Triose phosphate isomerase: the activity of this enzyme is present in a large excess and the reaction that is catalyses is already very close to equilibrium. Therefore, it is unlikely that increasing its activity will increase the concentration of glyceraldehyde-3-phosphate significantly, and so 3-PGA concentration will remain unchanged.

The preferred route for achieving an increase in 3-PGA concentration independently of any manipulation to decrease NAD-malic enzyme activity, is to increase the activity of phosphoglycerate kinase.

As well as the glycolytic pathway found in the cytosol, there is an equivalent sequence of reactions found within the plastids. Pyrophosphate:fructose-6-phosphate 1-phosphotransferase and PEP carboxylase are not found in the plastid, but there are isoforms of all of the other glycolytic enzymes present in the plastids in most plant species. Because the target enzyme for increased 3-PGA concentration, ADPglucose pyrophosphorylase, is located in the plastid, it may be beneficial to target the increase in 3-PGA concentration to this organelle. This can be achieved by specifically increasing the activity of, for example, phosphoglycerate kinase, in the plastid.

In general, strategies similar to those described for 3-PGA can be applied to any metabolites. The general problem of increasing metabolite concentrations through manipulation of enzyme activities has been considered theoretically (Small and Kacser 1994), although few attempts have been made to apply these approaches to plant systems. The reluctance to use such approaches stems at least partly from the generally held belief that metabolite concentrations are tightly regulated within cells and are therefore difficult to manipulate. Evidence herein demonstrates that contrary to those beliefs, manipulation of enzyme activities in glycolysis and the TCA cycle is effective to increase the starch content of plants.

The invention will now be further described in the Examples which follow.

EXAMPLES

Example 1

Reduction of NAD-ME activity in potato plants using antisense techniques

Transgenic potato lines (20 independent transformants) were produced by the method described in Hill *et al* 1996. Briefly, a cDNA clone encoding the 59 kDa subunit of NAD-ME was isolated from a potato cDNA expression library using affinity purified antibody that had been raised against purified potato NAD-ME (Winning *et al* 1994). The cDNA was cloned into a binary vector pBIN19 (Bevan 1984). The binary vector was introduced into *Agrobacterium* by direct transformation according to Höfgen and Willmitzer (1988). Potato stem explant transformation, using *Agrobacterium*-mediated gene transfer, was performed using the method of Twell and Ooms (1987) with the modifications of Sheerman and Bevan (1988). The cDNA was thus re-introduced into potato plants (cv. Desiree) in the antisense orientation, under the control of the 35S cauliflower mosaic virus promoter.

NAD-ME activity was determined by measuring the rate of conversion of pyruvate to malate by extracts of potato tubers; pyruvate concentrations were measured using a spectrophotometric assay. The starch content of potato tuber extracts was determined by measuring the amount of glucose released by digestion with amyloglucosidase; glucose concentrations were measured using a spectrophotometric assay.

To measure the rate of starch synthesis, discs were cut from stored tubers and incubated in the presence of ^{14}C-glucose for 2 hours. The rate of starch synthesis was estimated as the ^{14}C solubilised from starch by amyloglucosidase digestion.

Results are shown in Figures 2 to 4. Figure 2A shows the NAD-ME activity in a range of transgenic lines; controls were wild type (WT), untransformed or PA7 containing the vector DNA only. Figure 3 shows (A) the relationship between NAD-ME activity and starch content of

the tuber tissue; and (B) the relationship between NAD-ME activity and the rate of synthesis of starch by potato tuber discs. Values are the mean ± SE (n= 3-5). Figure 4 shows repeated measurements of starch content in tubers from lines with a range of NAD-ME activities. In Figures 3 and 4, each point represents a separate transgenic line. Together, these results show a good correlation between increased starch content and reduction in NAD-ME activity, in several independent transgenic lines, and a good correlation between increased starch content and flux of carbon into starch. The transgenic plants which gave the results in Figure 4 were from a different batch of plants to those giving the results in Figure 3. The figure 4 results confirm that the transgenic manipulation causes a reduction in the activity of NAD-ME, which in turn correlates with a substantial (two-fold) increase in the starch content of the tubers.

Preliminary data (not shown) indicates that no increase in starch is found in the leaves of these transgenic lines.

Example 2

Reduction of NAD-ME activity in a tuber-specific manner using the patatin promoter

In parallel with the production of plants with reduced NAD-ME activity in all tissues, as described in Example 1, transgenic plants were produced containing the antisense gene under the control of the potato B33 patatin promoter (Rocha-Sosa *et al* 1989). Figure 2B shows the NAD-ME activity in a range of these transgenic lines. The enzyme activity was measured as described above. A high starch phenotype is observed in these plants (data not shown).

Example 3

Reduction of NAD-ME activity

Transgenic potato lines were produced as described in Examples 1 and 2 and the relationship between NAD-malic enzyme and starch content in developing tubers was ascertained. Results for a typical batch of greenhouse plants are shown in Figure 5. Points on the graph represent individual tubers from a single batch of plants grown at the same time (Harvest No. 1). The line is fitted by linear regression and the correlation coefficient is shown in Table 1. The significant correlation shown by this batch of plants has been observed in other batches of plants grown independently (Table 1).

Table 1. Correlations between NAD-malic enzyme activity, 3-phosphoglyceric acid content and starch content in developing potato tubers. NAD-malic enzyme activity, 3-phosphoglyceric acid content and starch content were measured in developing tubers from a range of transgenic lines with decreased NAD-malic enzyme activity and wild type plants. Correlation coefficients were measured by linear regression and the significance of the correlation determined by a two-tailed significance test on the correlation coefficient. Values marked with a star are significant ($P<0.05$). nd = not determined.

Harvest	Sample size (No. of tubers)	Correlation coefficient (r) for comparison between		
		NAD-ME vs starch	NAD-ME vs 3-PGA	3-PGA vs starch
1	40	0.345*	0.358*	0.744*
2	20	0.314	0.453*	0.454*
3	26	0.510*	0.411*	0.539*
4	19	nd	nd	0.614*

The preliminary measurements of the rate of starch synthesis shown in Figure 3B were repeated. In particular, while the experiments described in Examples 1 and 2 were carried out with stored potato tubers, data now described is for developing potato tubers. Discs of tissue from developing tubers were supplied with radioactive (^{14}C) glucose and the metabolic fate of that glucose was investigated. The results are shown in Figure 6 for three of the lowest NAD-ME activity lines, and a transformed line with wild type NAD-malic enzyme activity. Tuber discs were taken from recently harvested developing tubers and incubated in the presence of ^{14}C-glucose for 2 hours. The tissue was killed and fractionated and the incorporation of ^{14}C into various components determined. Data is expressed as a percentage of the total ^{14}C metabolised and values are the mean of three replicates. Line T15 has a similar NAD-malic enzyme activity to wild type; lines 20, 21 and 22 have significantly reduced activities. The increased labeling of starch and decreased labeling of sucrose are consistent with there being a significant increase in the rate of starch synthesis in the tuber tissue with reduced NAD-malic enzyme activity.

Example 4

Increased 3-phosphoglyceric acid in potato tubers correlates with increased starch content

The content of 3-PGA in the transgenic potato tubers of Example 3 was measured. The result for a typical batch of plants is shown in Figure 7. Points represent individual tubers from a single batch of plants grown at the same time (Harvest No. 1). The lines are fitted by linear regression and the correlation coefficients are shown in Table 1. There is a strong negative correlation between the NAD-malic enzyme activity and the content of 3-PGA; the starch content shows a strong positive

correlation with that of 3-PGA. The NAD-malic enzyme activity/3-PGA correlation and the starch/3-PGA correlation has been observed in batches of plants grown independently (Table 1).

Example 5

Isolation of cDNAs encoding NAD-malic enzyme

(a) Using the published sequence of the two potato cDNAs that encode subunits of NAD-malic enzyme (Winning *et al* 1994) identify sequences that are conserved between the two subunits at either end (5' and 3') of the cDNAs. Synthesise oligonucleotides corresponding to the two sequences for use as primers in the polymerase chain reaction (PCR; see, for example, Grof *et al* 1995).

(b) Produce a cDNA library from mRNA isolated from the tissue to be manipulated from the species of interest. It is important to use mRNA from the target tissue, so that cDNAs corresponding to the correct isoform of NAD-malic enzyme can be isolated.

(c) Use the primers produced in (a) to amplify cDNAs from the library produced in (b) that contain DNA nucleotide sequences which encode corresponding to NAD-malic enzyme. The identity of the sequences as encoding NAD-malic enzyme is confirmed by comparison with the sequence from potato (Winning *et al* 1994).

Example 6

Production of potato plants with short-sense suppression of NAD-malic enzyme activity in their tubers

(a) Subclone a 50-100 nucleotide fragment of the cDNA encoding the 59 kDa subunit of NAD-malic enzyme into a modified binary vector in the forward orientation. The cDNA fragment is inserted immediately downstream of the B33 patatin promoter (Rocha-Sosa *et al* 1989) which confers tuber-enhanced expression on the cDNA fragment. Expression of such short-sense genes has been shown to lead to reduced

activity of the target enzyme (Cameron and Jennings 1991; Que *et al* 1997). As well as the cDNA and the patatin promoter the binary vector also contains, between the T-DNA borders, a cDNA encoding phosphomannose isomerase.

(b) Transfer the binary vector produced in (a) into *Agrobacterium tumefaciens* (Strain LBA4404) using the direct transformation method of Höfgen and Willmitzer (1988).

(c) Transfer the T-DNA from the Agrobacterium into potato stem explants using the co-cultivation method according to Twell and Ooms (1987) with the modifications of Sheerman and Bevan (1988).

(d) Identify transformed plants through their ability to grow in the presence of mannose. Mannose is normally toxic to plant cells, but the transformed plants will be able to tolerate it by virtue of the presence of the phosphomannose isomerase sequence.

Example 7

Production of maize plants with short-sense suppression of NAD-malic enzyme activity in the endosperm of their seed

(a) Subclone a 50-100 nucleotide fragment of a cDNA encoding the 59 kDa subunit of NAD-malic enzyme from maize, obtained as in Example 5 above, into a plasmid. The cDNA fragment is inserted immediately downstream of the zein promoter (Schernthaner *et al* 1988) which confers endosperm-enhanced expression on the cDNA fragment. Expression of such short-sense genes has been shown to lead to reduced activity of the target enzyme (Cameron and Jennings 1991, Que *et al* 1997). As well as the cDNA and the zein promoter the plasmid also contains a cDNA encoding phosphinothricin acetyl transferase driven by the constitutive CaMV 35S promoter. The latter gene confers resistance to the glufosinate ammonium.

(b) Transfer the plasmid into embryogenic maize suspension culture cells using the silicon carbide whiskers transformation method (Frame *et al* 1994) and regenerate plants from the suspension culture cells according to Frame *et al* (1994).

(d) Identify transformed plants through their ability to grow in the presence of the herbicide glufosinate ammonium.

Example 8

Production of oil-seed rape (*Brassica napus*) plants with short-sense suppression of NAD-malic enzyme activity in the endosperm of their seed.

(a) Subclone a 50-100 nucleotide fragment of a cDNA encoding the 59 kDa subunit of NAD-malic enzyme from oil seed rape, obtained as in Example 5 above, into a modified binary vector in the forward orientation. The cDNA fragment is inserted immediately downstream of a seed-specific oleosin promoter (Keddie *et al* 1994) which confers seed-enhanced expression on the cDNA fragment. Expression of such short-sense genes has been shown to lead to reduced activity of the target enzyme (Cameron and Jennings 1991, Que *et al* 1997). As well as the cDNA and the oleosin promoter the binary vector also contains, between the T-DNA borders, a cDNA encoding phosphomannose isomerase.

(b) Transfer the binary vector produced in (a) into *Agrobacterium tumefaciens* (Strain LBA4404) using the direct transformation method of Höfgen and Willmitzer (1988).

(c) Transfer the T-DNA from the *Agrobacterium* into leaf discs using the co-cultivation method .

(d) Identify transformed plants through their ability to grow in the presence of mannose. Mannose is normally toxic to plant cells, but the transformed plants will be able to tolerate it by virtue of the presence of the phosphomannose isomerase sequence.

Example 9

Increasing the concentration of 3-PGA in potato tubers by increasing the activity of cytosolic phosphoglycerate kinase.

(a) Obtain a cDNA encoding phosphoglycerate kinase from a plant species other than potato and subclone it into a modified binary vector in the forward orientation. The cDNA encoding this enzyme from tobacco has been isolated (Bringloe *et al* 1996) and is freely available. The cDNA is inserted immediately downstream of the B33 patatin promoter (Rocha-Sosa *et al* 1989) which confers tuber-enhanced expression on the cDNA. As well as the cDNA and the patatin promoter the binary vector also contains, between the T-DNA borders, a cDNA encoding phosphomannose isomerase.

(b) Transfer the binary vector produced in (a) into *Agrobacterium tumefaciens* (Strain LBA4404) using the direct transformation method of Höfgen and Willmitzer (1988).

(c) Transfer the T-DNA from the *Agrobacterium* into potato stem explants using the co-cultivation method according to Twell and Ooms (1987) with the modifications of Sheerman and Bevan (1988).

(d) Identify transformed plants through their ability to grow in the presence of mannose. Mannose is normally toxic to plant cells, but the transformed plants will be able to tolerate it by virtue of the presence of the phosphomannose isomerase sequence.

Example 10

Increasing the concentration of 3-PGA in the amyloplasts of potato tubers by increasing the activity of plastidic phosphoglycerate kinase

(a) Obtain a cDNA encoding phosphoglycerate kinase from a species other than potato and subclone it into a modified binary vector in the forward orientation. The cDNA encoding the plastid isoform of this enzyme from tobacco has been isolated (Bringloe *et al* 1996) and is freely

available. The cDNA is inserted immediately downstream of the B33 patatin promoter (Rocha-Sosa *et al* 1989) which confers tuber-enhanced expression on the cDNA. Between the cDNA and the promoter the plastid targeting sequence from tobacco ribulose bisphosphate carboxylase is inserted as a translational fusion with the cDNA. This will ensure that the tobacco phosphoglycerate kinase that is produced in the potato tuber will be directed to the amyloplast. As well as the cDNA and the patatin promoter the binary vector also contains, between the T-DNA borders, a cDNA encoding phosphomannose isomerase.

(b) Transfer the binary vector produced in (a) into *Agrobacterium tumefaciens* (Strain LBA4404) using the direct transformation method of Höfgen and Willmitzer (1988).

(c) Transfer the T-DNA from the *Agrobacterium* into potato stem explants using the co-cultivation method according to Twell and Ooms (1987) with the modifications of Sheerman and Bevan (1988).

(d) Identify transformed plants through their ability to grow in the presence of mannose. Mannose is normally toxic to plant cells, but the transformed plants will be able to tolerate it by virtue of the presence of the phosphomannose isomerase sequence.

Example 11

Increasing the concentration of 3-PGA in the cytosol of potato tubers with minimal effects on the flux through glycolysis

This method is based on the theoretical approach proposed by Kacser and Acerenza (1993). In order to minimise the effects on metabolic flux the activity of the enzyme that produces 3-PGA, phosphoglycerate kinase, is increased and the activity of the enzyme that consumes it, phosphoglycerate mutase, is decreased. The increase in phosphoglycerate kinase is produced as described in Example 9, while the decrease in enolase activity can be achieved using a similar approach to

that described in Example 6 having obtained a cDNA encoding enolase from potato.

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CLAIMS

1. A method for producing starch comprising the steps of:

a) providing a plant, which plant is genetically modified so as to have increased starch content as a result of a change in the concentration of one or more of the metabolites which are intermediates in glycolysis and the TCA cycle in the plant; and

b) harvesting starch-containing material from the plant.

2. The method according to claim 1, wherein the one or more metabolites includes one or more metabolites from the group consisting of malate, oxaloacetate, phospho(enol)pyruvate and 3-phosphoglycerate.

3. The method according to claim 2, wherein the one or more metabolites includes 3-phosphoglycerate.

4. The method according to any one of claims 1 to 3, wherein the plant is modified to achieve an increase or decrease in the activity of one or more enzymes of the plant, so as to change the concentration of the one or more metabolites.

5. The method according to claim 4, wherein the one or more enzymes include an enzyme which takes part in glycolysis or the TCA cycle as shown in figure 1A.

6. The method according to claim 4 or claim 5, wherein the activity of NAD-malic enzyme (NAD-ME) is decreased.

7. The method according to any one of claims 4 to 6, wherein the plant is a transgenic plant in which expression of a heterologous DNA sequence effects an increase or a decrease in the enzyme activity.

8. The method according to any one of claims 1 to 7, wherein the plant is a starch-storing plant.

9. The method according to claim 8, wherein the starch content is preferentially increased in starch-storing tissues of the plant.

10. A genetically modified plant having enhanced starch-producing capability as a result of a change in the concentration of one or more of the metabolites which are intermediates in glycolysis and the TCA cycle in the plant, with the proviso that potato plants having reduced NAD-ME activity by means of antisense techniques, are excluded.

11. The modified plant according to claim 7, wherein the one or more metabolites includes one or more metabolites from the group consisting of malate, oxaloacetate, phospho(enol)pyruvate and 3-phosphoglycerate.

12. The modified plant according to claim 10 or claim 11, wherein the one or more metabolites includes 3-phosphoglycerate.

13. The modified plant according to any one of claims 9 to 12, wherein the plant is modified to achieve an increase or decrease in the activity of one or more enzymes of the plant, so as to change the concentration of the one or more metabolites.

14. The modified plant according to claim 13, wherein the one or more enzymes include an enzyme which takes part in glycolysis or the TCA cycle as shown in Figure 1A.

15. The modified plant according to claim 13 or claim 14, wherein the activity of NAD-ME is decreased.

16. The modified plant according to any one of claims 13 to 15, wherein the plant is a transgenic plant in which the expression of a heterologous DNA sequence effects an increase or a decrease in the enzyme activity.

17. The modified plant according to any one of claims 9 to 16, wherein the plant is a starch-storing plant.

18. The modified plant according to any one of claims 9 to 17, wherein the starch-producing capability is preferentially enhanced in starch-storing tissues of the plant.

19. A plant cell, genetically modified so as to be capable of regeneration into a modified plant according to any one of claims 8 to 18.

20. The use of a modified plant according to any one of claims 8 to 18, for the production of starch-containing material.

21. The use of a modified plant cell according to claim 19, for regenerating a plant for the production of starch-containing material.

22. The use of a starch-storing plant which is genetically modified so as to decrease NAD-ME activity, for the production of starch-containing material.

23. The use of a plant cell of a starch-storing plant, said cell genetically transformed so as to decrease NAD-ME activity in a plant regenerated from the plant cell, for regenerating a plant for the production of starch-containing material.

24. A recombinant double-stranded DNA molecule comprising a promoter which functions in plants, which promoter is capable of functioning preferentially in starch-storing tissues of plants, said promoter operatively linked to a DNA sequence capable of reducing NAD-ME activity in plants.

25. A recombinant double-stranded DNA molecule comprising a promoter which functions in plants operatively linked to a DNA sequence capable of reducing NAD-ME activity in plants, with the proviso that the DNA sequence is not derived from a potato plant.

26. The recombinant DNA molecule according to claim 24 or claim 25, wherein the DNA sequence capable of reducing NAD-ME activity comprises a sequence encoding a subunit of NAD-ME, in antisense orientation.

27. The recombinant DNA molecule according to claim 24 or claim 25, wherein in potato, the promoter is preferentially active in tubers.

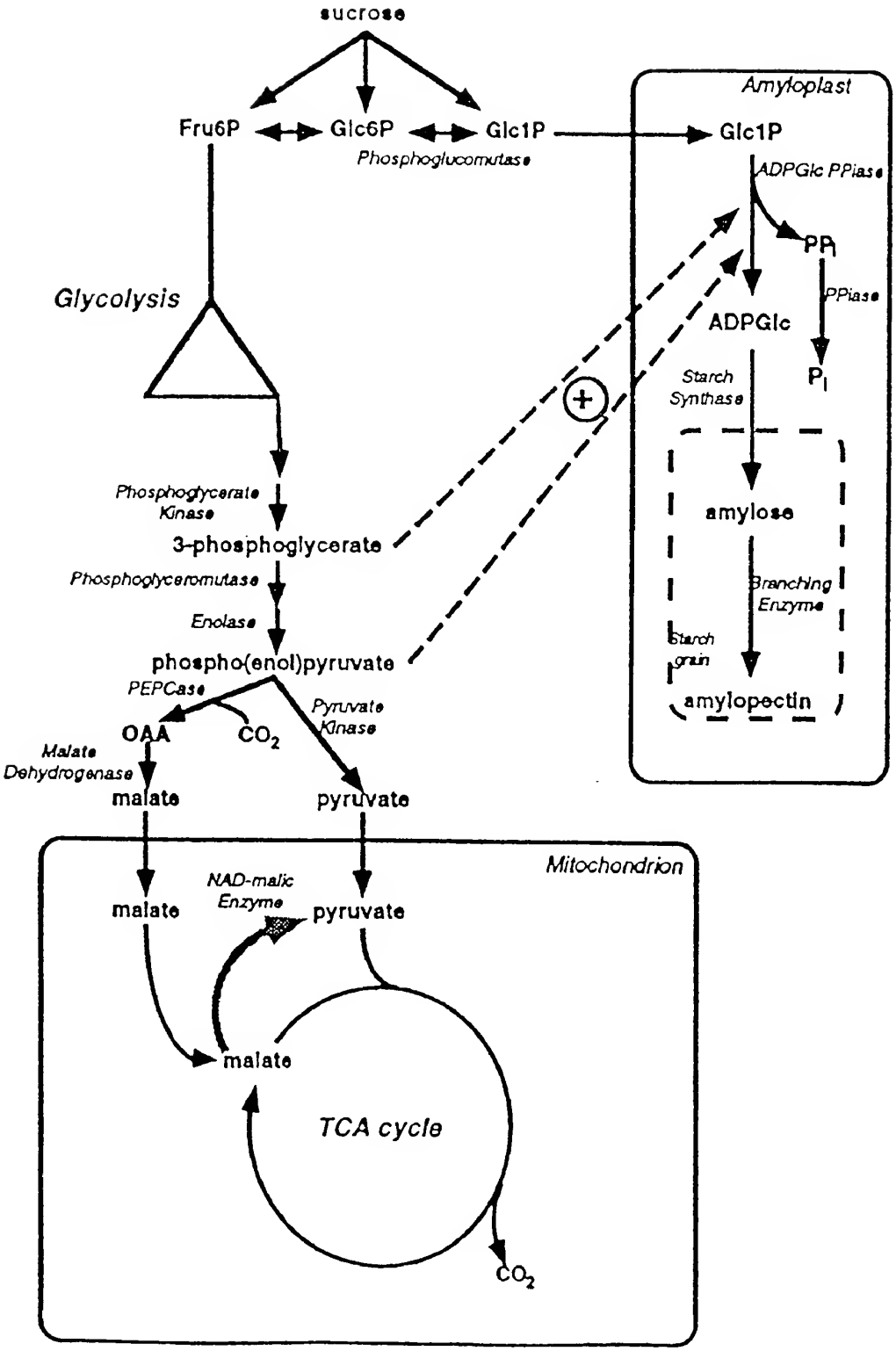
28. The recombinant DNA molecule according to claim 27, wherein the promoter is or is derived from the patatin promoter.

29. The recombinant DNA molecule according to any one of claims 24 to 28, contained in a vector suitable for transforming plant cells.

30. A plant cell transformed with the recombinant DNA molecule according to any one of claims 24 to 29.

31. A plant consisting of plant cells according to claim 30, wherein NAD-ME activity is preferentially decreased in starch-storing tissues of the plant.

Figure 1A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ADPGlc
Starch Synthase
Pi
amylose
Branching Enzyme
Starch grain
amylopectin
Phosphoglycerate Kinase
3-phosphoglycerate
Phosphoglyceromutase
Enolase
phospho(enol)pyruvate
PEPCase
OAA
CO2
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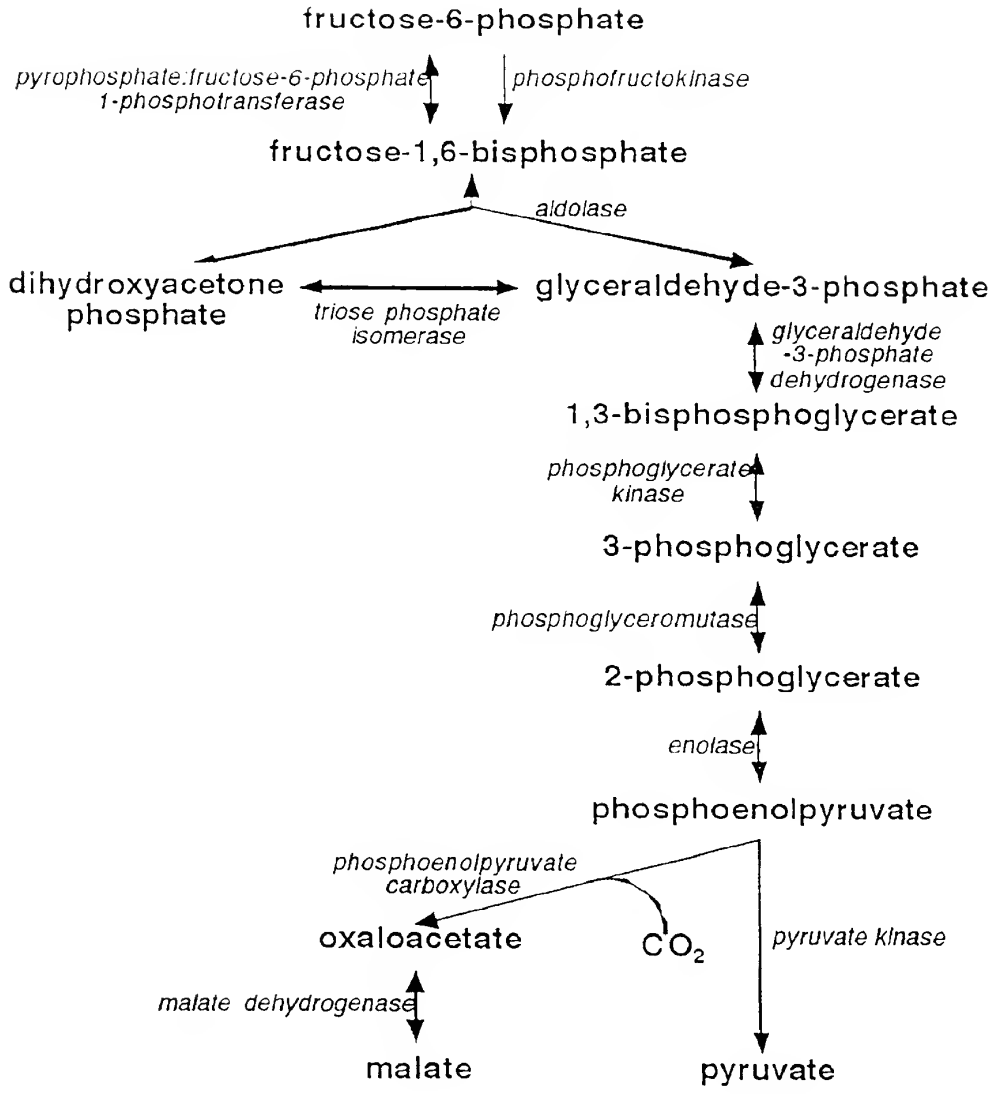
pyruvate
malate
TCA cycle
CO2

Figure 1B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fructose-1,6-bisphosphate
aldolase
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glyceraldehyde-3-phosphate
glyceraldehyde -3-phosphate dehydrogenase
1,3-bisphosphoglycerate
phosphoglycerate kinase
3-phosphoglycerate
phosphoglyceromutase
2-phosphoglycerate
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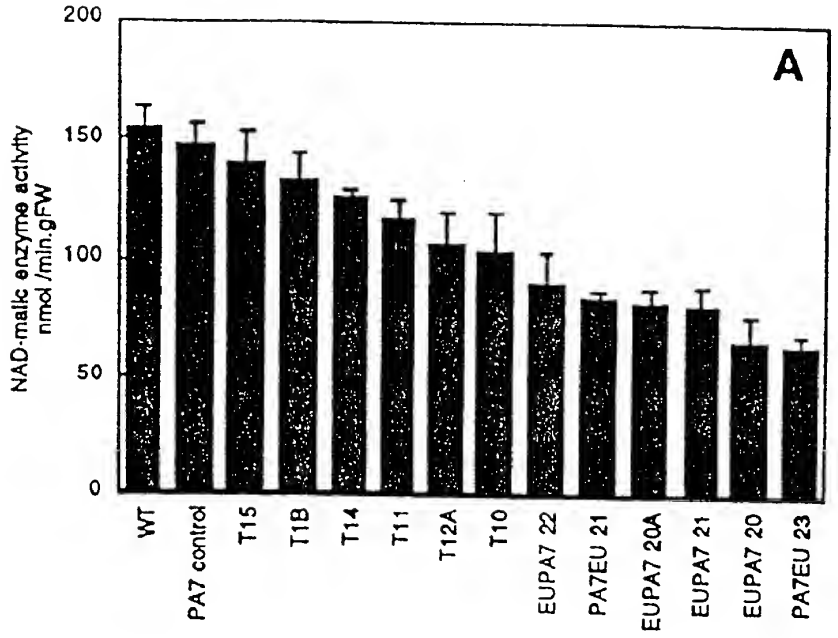
Figure 2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100
50
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PA7 control
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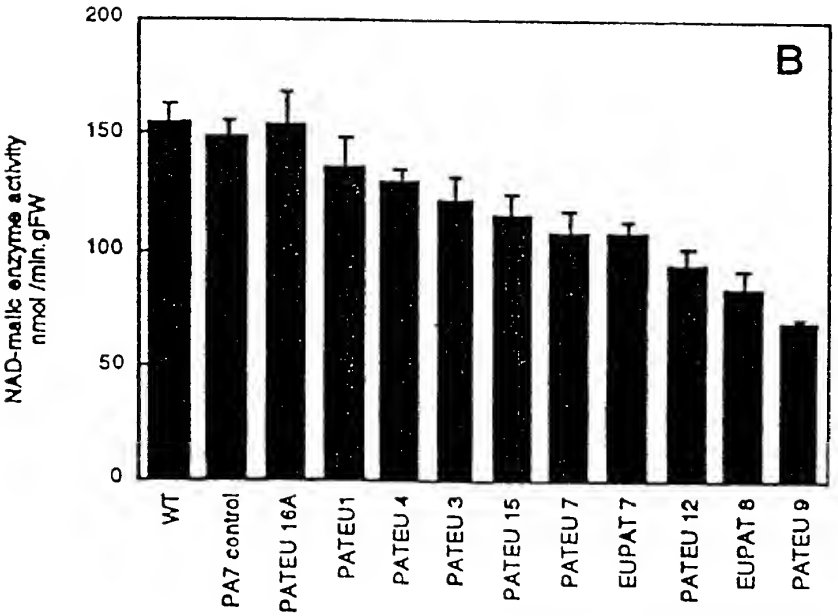
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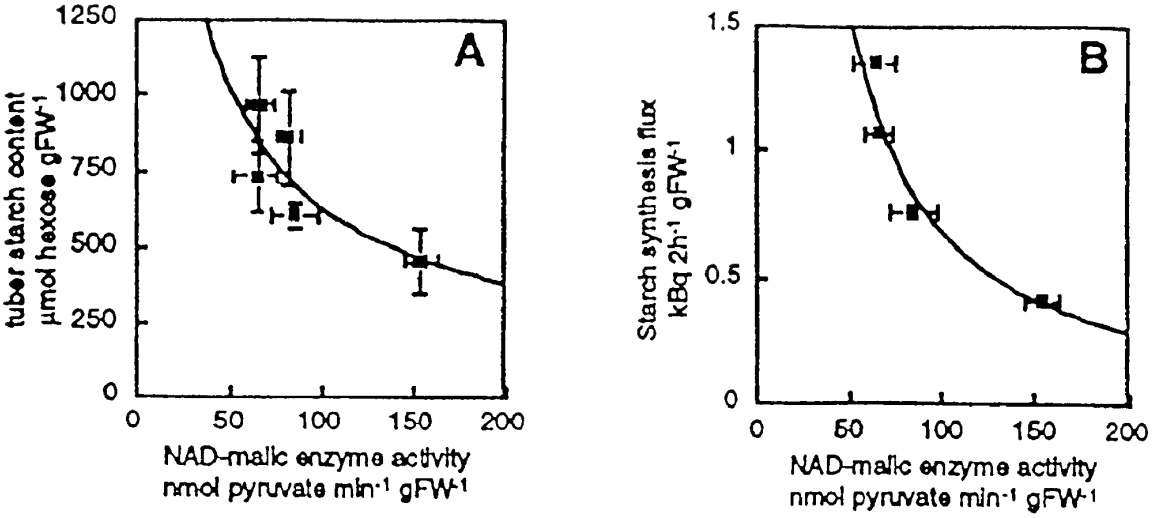
Figure 3



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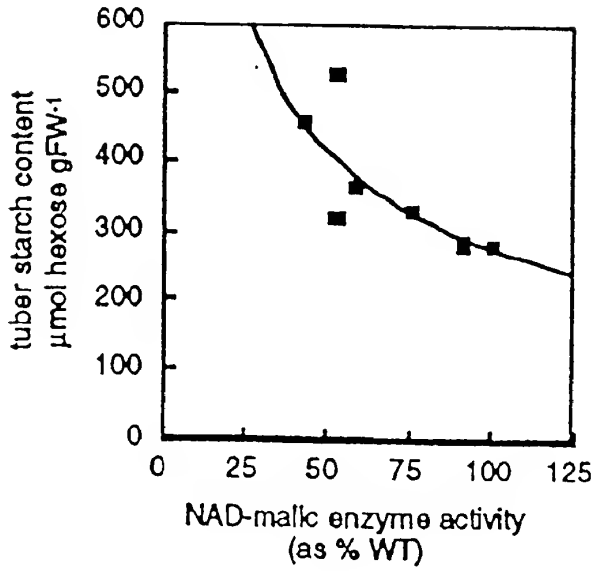


Figure 5

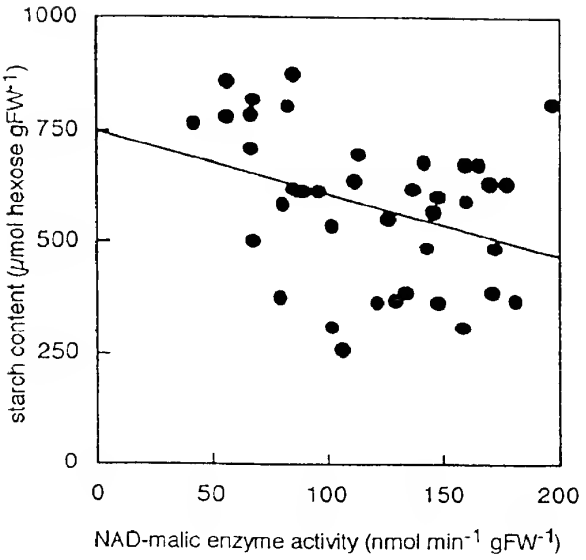
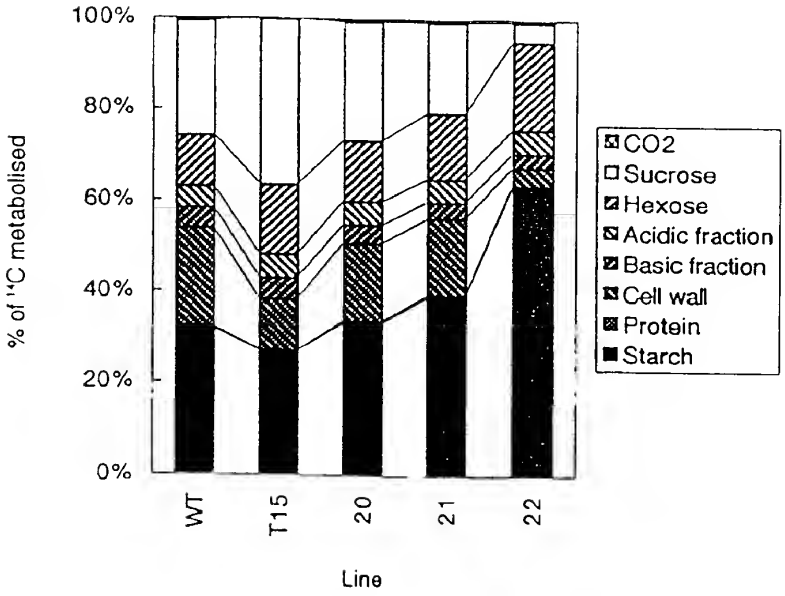
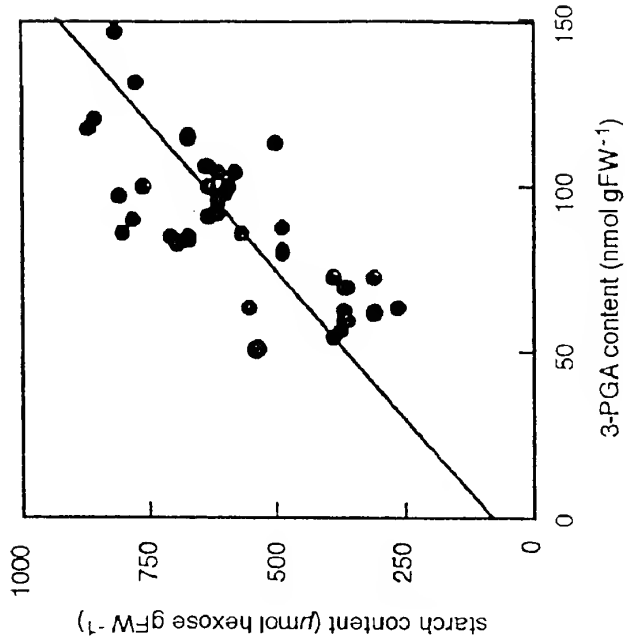


Figure 6




starch content (μmol hexose gFW -1)
1000
750
500
250
0
0
50
100
150
3-PGA content (nmol gFW-1)

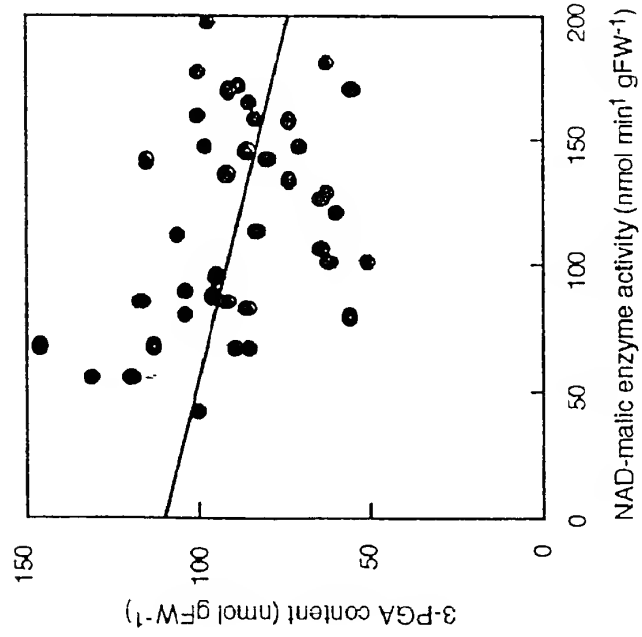

3-PGA content (nmol gFW-1)
150
100
50
0
0
50
100
150
200
NAD-malic enzyme activity (nmol min1 gFW-1)

Figure 7

Figure 8

59 kDa:

```
CTCGAATTCA AAGTTTTGTT GATTCAATTT TTACGCATTT TTTTATTTAT ATAAGGAGCG   60
TGCAATGTGG AGAGTGGCTC GATCTGCGGC GTCGACTTTC CGCCGTACGC GGCGGTTATC  120
GACGGCGATT TCAGCTCCAT GTATCGTACA CAAGCGCGGT GCTGATATTC TTCATGATCC  180
TTGGTTCAAT AAGGACACGG GATTCCCAAT GACGGAAAGA GATCGATTGG GTCTGCGCGG  240
TCTCCTCCCA CCTCGTGTAA TATCATTTGA ACAGCAATAT GACCGCTTCA TGGAGTCATT  300
TCGGTCACTT GAGAAAAATA CTGAGGGTCA ACCAGACAGT GTTGTTTCGT TAGCAAAATG  360
GAGGATCTTA AACAGGCTGC ATGACAGGAA TGAGACACTG TACTACCGAG TTCTAATTGA  420
CAATATCAAA GATTTTGCTC CCATAATATA CACTCCAACA GTAGGATTGG TTTGCCAAAA  480
CTACTCTGGT TTGTTTAGAC GTCCACGTGG CATGTATTTC AGTGCCAAAG ATAAGGGAGA  540
GATGATGTCA ATGATCTTCA ACTGGCCTTC TACTCAGGTA GACATGATTG TGCTGACAGA  600
TGGAAGTCGT ATTCTTGGCC TTGGCGATCT TGGAGTTCAG GGGATAGGTA TACCAATTGG  660
AAAACTTGAC ATGTATGTAG CAGCTGCTGG TATCAACCCA CAAAGAGTGC TCCCAGTTAT  720
GCTTGACGTC GGGACCAACA ATCAGAAGCT TCTAGAAGAT CCTCTTTATC TTGGACTGCG  780
ACAACCTAGG TTGGAAGGAG AAGAATATTT GTCAATAGTT GATGAATTTG TGGAAGCTGT  840
TCATGCTCGT TGGCCAAAGG CTGTTGTGCA GTTTGAGGAT TTTCAAGCAA AGTGGGCTTT  900
TGAGACACTG GATCGCTATC GGAAAAAGTT TTGCATGTTC AATGATGATA TACAGGGAAC  960
AGCTGGTGTT GCTCTTGCTG GGCTATTAGG AACTGTTAGG GCACAGGGCC GGCCACTGAC 1020
TGATTTTGCC AACCAAAAGA TAGTGGTAGT TGGAGCAGGA AGTGCGGGGC TTGGTGTTCT 1080
TAAGATGGCA TTACAAGCCG TTTCAAGAAT GACTGGACCA TCAGCAGATC CCCACTTTTT 1140
TCTTCTGGAT AAAAATGGTC TTATCACAAA GGATAGGAAA GATATTGATC CAGCAGCATT 1200
ACCATTTGCT AAAGCCCATC ACGAGATTGA GGGGCTAGGA CTACAGGAGG GAGCAGGGCT 1260
CGCTGAAGTG GTCAAAAAGG TGAAGCCTCA TGTACTTCTT GGTTTGTCAG GAGTTGGAGG 1320
CATATTTCAT GAGGAGGTGC TGAGGGCCAT GAAAGAGTCA GATTCTGTTA GACCTGCAAT 1380
TTTCGCCATG TCAAATCCTA CAAACAATGC TGAATGCTGT CCTGTTGATG CTTTCAAGCT 1440
TGCTGGAGAA GATATTGTTT TTGCTAGTGG GAGTCCCTTT GCAAATGTTG ATCTAGGGAA 1500
TGGTAAGATA GGCCATGTAA ATCAAGCGAA TAACATGTAT CTCTTCCCTG GCATTGGGCT 1560
AGGAGCTCTT CTTTCTGGCG CTCGAAACAT AAGTGATACA ATGTTAGAAG CTGCTGCTGA 1620
GTGCCTTGCT TCTTACATGT CCGATGATGA AATCAATAGA GGAATCTTGT ATCCATCCAT 1680
TGATGATATT CGGGATATTA CAGCAGAGGT GGGAGCTGCA GTTTTACGGG CTGCTGTGGC 1740
TGAAGACCTG GCAGAAGGTC ATGGTGATGT TGGGGTAAAA GAGTTGCAGC ATATGTCAAA 1800
GGAAGAGACC ATTGAACACG TGAGACAAAA TATGTGGTAT CCCGTCTATG GCCCTCTTGT 1860
TCACGAATAG TGAAGCACTT CCTGGGTTGA GATCACAGGT CCACGAGAAG TGGAGTATGA 1920
TCTGGTCGTC TAAATTGCCT GTAAAGGTAA AAAAAAATCG CGAACCTGAC TGTATTAGGT 1980
GTACAATTAC AAGGACAAAT GTTGCTGAAC CACGAAAGAG ACAAGGGCTC TTCAAATATT 2040
TCATTTTTTT GTTGTTAGCC ATTCTTGTTT ATCTCATGAA GCATATGTAA CCTTTGATTT 2100
GACTAGGAGA TGTATTTGTT ATCTCAGAAT AAAAGCTGAT ACTTTCTCTC AGCACCACCA 2160
CCTATTGAAC TGTATTCATT TTTGTGGT                                     2188
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Figure 8 continued

62kDa

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CTTCACTGTC TTTCTTCGAT CCAGAAAAAT GGCGATTTTT TCCAATCAAA TGCGACTTTC   60
CTCAACTCTT CTGAAGCGTT TACACCAAAG AGTTGCTGCT GCTGTGAATT CATCTTCTTC  120
AAGAAATTTT ACTACAACGG AAGGTCACCG TCCCACCATT GTTCACAAGC GGAGTCTTGA  180
TATTCTTCAC GATCCATGGT TTAACAAGGG AACAGCATTC TCATTTACTG AACGTGATCG  240
TCTTCACATT CGTGGCTTAC TGCCTCCAAA CGTGATGTCT TTTGAACAAC AAATTGCACG  300
GTTCATGGCT GACTTGAAGA GGCTTGAAGT ACAAGCTAGA GATGGTCCAT CTGACCCATA  360
TGTTTTGGCC AAGTGGCGCA TACTTAATCG CTTGCACGAT AGAAATGAGA CTTTGTACTA  420
CAAGGTTCTA ATGGAAAATA TTGAGGAATA TGCACCCATA GTGTATACTC CTACAGTTGG  480
TCTTGTTTGC CAGAAGTACA GTGGATTGTT CAGACGTCCG AGGGGAATGT ACTTCAGTGC  540
CGAAGATCGT GGGGAAATGA TGTCAATGGT TTACAATTGG CCAGCTGATC AGGTTGACAT  600
GATTGTTGTA ACTGATGGAA GTAGAATATT GGGTCTTGGA GATCTCGGAA TTCAGGGAAT  660
TGGTATTGCA ATTGGGAAGC TGGATCTGTA TGTGGCTGCT GCTGGGATCA ACCCTCAAAG  720
AGTACTGCCT GTAATGATTG ATGTTGGAAC TGACAACGAG AATCTCTTGA AAGACCCTTT  780
ATATTTGGGA CTGCAAGATC ATCGGCTTGA CGGAGAGGAG TATATTGAAG TAATTGATGA  840
ATTTATGGAG GCTGTTTTTA CTCGTTGGCC CCATGTGATT GTGCAGTTTG AAGATTTTCA  900
AAGCAAGTGG GCCTTTAAGC TGTTGCAGCG TTATAGAAAC AACTACAGAA TGTTTAATGA  960
TGATATCCAG GGAACTGCAG GAGTTGCTAT TGCTGGTCTT CTGGGAGCAG TAAGAGCACA 1020
GGGAAGACCA ATGATTGATT TCCCAAAAAT GAAGATTGTG GTGGCGGGTG CTGGAAGTGC 1080
AGGAATAGGG GTTCTTAATG CTGCTAGGAA GACAATGGCA AGAATGCTAG GGAATACTGA 1140
AATTGCTTTT GAAAGTGCAA GGAGTCAGTT CTGGGTGGTT GATGCCAAGG GATTAATTAC 1200
TGAGGCACGT GAAAATGTTG ATCCCGATGC TCGCCCGTTT GCCAGAAAGA TCAAAGAGAT 1260
TGAACGTCAA GGTCTAAGTG AAGGTGCAAC TCTCGCGGAA GTGGTACGCG AAGTGAAGCC 1320
AGATGTACTT CTTGGTTTAT CTGCTTGTGG AGGCTTGTTT TCAAAAGAGG TACTGGAAGC 1380
CCTTAAGCAT TCAACTTCAA CCCGGCCTGC AATTTTTCCA ATGTCAAACC CAACGAGAAA 1440
TGCTGAATGC ACACCTGAGG AAGCATTTTC TATTCTCGGT GAGAACATTA TATTTGCAAG 1500
TGGAAGCCCA TTCAAGGATG TGGATCTTGG AAACGGTCAT GTTGGACACT GCAACCAAGC 1560
AAACAACATG TTCCTTTTTC CTGGAATAGG ACTTGGTACT CTTCTGTCTG GATCTAGAAT 1620
CGTTTCAGAT GGCATGTTAC AGGCTGCAGC TGAGTGTTTG GCTGCATATA TTACAGAGGA 1680
GGAGGTACTC AAGGGGATTA TATACCCTTC GATATCCAGG ATACGCGATA TAACAAAGGA 1740
GGTGGCTGCA GCTGTGGTGA AAGAAGCTAT AGAAGAGGAT CTGGCTGAAG GATACCGTGA 1800
AATGGACAGC CGAGAGTTAC GGAAACTTGA TGAGGCACAA ATATCTGAGT TCGTGGAGAA 1860
CAATATGTGG AGTCCTGATT ATCCAACACT GGTTTACAAG AAAGATTGAT CATCTATAGC 1920
TGCTTGCAAA GCTTCATCTT TTCATGTTTC TCGCAACATC AGCAAGGGCG TGATGGATCA 1980
AATACGATCA TGGACATTTT GTTACATGAT GGGATCATGT AAATTTAACA TCCAGTAATA 2040
TCCCATTCTT CCAACATTTG ATTGGCTGAG GACTTCTTGA TATTATAGCT TTTGTTGAGT 2100
AGTCCATAAA TTGGTTGTCT GTAAAAGTGA ATAAATCCCC TTCTCCTTTC AATTCTGATT 2160
TTGAGCTTCG TCAACGCAGA CGATGTAGTC TTACGGGCTG TTGCTGCACA GATGTACTAT 2220
AAATTGCTCT GTGATGTAGA TGTTGTGTAA A                                  2251
```

INTERNATIONAL SEARCH REPORT

Internc ·al Application No
PCT/uB 97/03245

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/11 C12N5/10 A01H5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILL S. ET AL.: "Role of NAD+-dependent 'malic' enzyme and pyruvate dehydrogenase complex in leaf metabolism" BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 24, August 1996, pages 743-746, XP002059242 cited in the application see the whole document ---	25,26, 29,30
X	RIESMEIER J. ET AL.: "Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants" PNAS,U.S.A., vol. 90, no. 13, 1 July 1993, pages 6160-6164, XP002059243 see the whole document ---	1-4,7,8, 10-13, 16,17,19
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[X] Patent family members are listed in annex.

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1

Date of the actual completion of theinternational search	Date of mailing of the international search report
18 March 1998	27/03/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Kania, T

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 5 498 831 A (BURGESS DIANE G ET AL) 12 March 1996 see the whole document	1-31
A	EP 0 455 316 A (INST GENBIOLOGISCHE FORSCHUNG) 6 November 1991 see the whole document	1-31
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A	HERBERS K ET AL: "Manipulating metabolic partitioning in transgenic plants" TRENDS IN BIOTECHNOLOGY, vol. 6, no. 14, June 1996, page 198-205 XP004035774 cited in the application see the whole document	1-31

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